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Positional effects of tRNA genes: transfer RNA genes as chromatin boundaries in Saccharomyces cerevisiae

Tiffany Anne Simms
Louisiana State University and Agricultural and Mechanical College, tsimms1@lsu.edu

POSITIONAL EFFECTS OF TRNA GENES: TRANSFER RNA GENES AS CHROMATIN BOUNDARIES IN *SACCHAROMYCES CEREVISIAE*

A Thesis

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ABSTRACT

Recently, much evidence has been brought forth into the scientific community supporting the idea that RNA Polymerase III transcribed regions of DNA may serve as chromosomal landmarks for silencing. Transfer RNA genes are known to involve themselves in several extra-transcriptional functions within the chromosome, including the pausing of replication forks, Ty element integration, tRNA position effects (repression of neighboring genes), acting as a barrier to the spread of heterochromatin, and over-riding nuclosome positioning sequences. Our results suggest that many tRNA genes may serve these functions as well as exhibiting behavior similar to metazoan insulators. Also, ETC (Extra $TF_{III}C$) sites within *Saccharomyces cerevisiae*, which bind only $TF_{III}C$ may also act as barriers or insulators. Our results support the idea that extra-transcriptional functions of RNA Polymerase III factors may be widespread and important contributors to genome biology.

CHAPTER 1: INTRODUCTION

In principle, every step in the pathway that leads from DNA to protein could, in theory, be regulated. There is a myriad of choices that a cell has available to it in order to regulate the production of a gene product. The most obvious, efficient, and cost-effective way to control the protein product of a gene is to control the initiation of RNA transcription. Each gene's transcription is controlled by DNA regulatory elements close to the site of transcription initiation. Some of these sites are very complex and respond to a variety of signals which they must interpret to determine whether or not to express the neighboring gene. Other regulatory regions are simple and can be activated by a single signal. Genes must be both positively and negatively regulated. In positive regulation, an activator protein binds and promotes transcription. In negative regulation, a repressor protein binds and prevents transcription. However, these simplified models of gene regulation apply only in principle to most eukaryotic genes.[1]

Eukaryotic gene regulation can become quite complex because of several factors. Eukaryotic genes are often regulated by proteins that can act even when bound relatively far from the transcription initiation site, and often there may be many regulatory elements that control a single promoter. RNA polymerase II, which transcribes all protein-encoding genes, requires that a set of transcription factors be bound to the DNA in a specific order prior to transcription initiation. These transcription factors bind the DNA sequence specifically, therefore allowing for a sort of throttle control on the rate of transcription initiation. The final layer of eukaryotic gene regulation is that eukaryotic DNA is packaged into chromatin, which can provide additional opportunities for regulation that are not available to prokaryotes.[1]

Eukaryotes also use activators and repressors to regulate gene expression, though they are used in different ways. Eukaryotic DNA sequences that bind to activators were originally called enhancers because they enhanced transcription levels. It was also discovered that enhancers could be located thousands of base pairs away from the promoter that they were acting upon. Enhancers can also influence transcription regardless of their location in relation to the promoter, whether they lie upstream or downstream. The promoter is where the transcription factors assemble and, subsequently, the polymerase assembles. In eukaryotes, some enhancers and promoters are separated by a distance of over 50,000 base pairs. Though much of this DNA is not recognized by the regulatory proteins, it is thought that this spacer DNA may allow for flexibility that allows interaction of enhancers and promoters. Also, since eukaryotic DNA is packaged into chromatin, chromosomes are thereby compacted.[1]

Nucleosomes are the fundamental unit of chromatin, consisting of an octamer of the four core histones: H2A, H2B, H3, and H4. There are variants of the core histone proteins that can serve important functions with regard to gene regulation. Each nucleosome is composed of two H2A/H2B dimers and one H3/H4 tetramer. One hundred forty-seven base pairs of DNA wrap around each nucleosome. The nucleosomes compact the genome within the nucleus, while also playing an important part in the expression of the underlying DNA. There are many post-translational modifications that occur on each of the core histone proteins, each playing its own role in gene regulation. Histones can be methylated on lysine and arginine residues, acetylated on lysine residues, phosphorylated on serine residues, or ubiquinated. Most of these modifications occur on

the amino-terminal tails of the histone proteins which extend from the core nucleosome. These modifications influence the binding of other chromatin proteins.[2]

Chromatin can be organized into domains of transcriptional activity by function and structural characteristics. There are two classes of chromatin: heterochromatin and euchromatin. Heterochromatin (condensed chromatin), which is packaged into a compact structure, is defined as transcriptionally inactive, generally gene poor, and having hypoacetylated nucleosomes. Euchromatin (decondensed chromatin), which is packaged less compactly than heterchromatin, is defined as being generally transcriptionally permissive, gene rich, and having hyperacetylated nucleosomes. These chromosome arrangements and structures are heritable and are now understood as being critical to regulating the expression of inducible and developmental genes.[2]

The compaction of chromatin proceeds in a step-wise manner, spreading along the chromosome as it compacts. In yeast, heterochromatin initiates at silencer sequences which bind Abf1 (autonomously replicating sequence binding factor) and Rap1 (repressor-activator protein). These silencer sequences also bind ORC (the origin recognition complex), which in turn recruits multiple Sir (silent information regulator) proteins, creating a Sir protein complex. Sir2p is a histone deacetylase and is recruited by interacting with Sir4p. Sir2p initiates heterochromatin propagation by deacetylating the neighboring nucleosomes. After these nucelosomes are deacetylated, Sir3p binds to the histone tails with higher affinity. Binding of Sir3p recruits additional Sir2p/Sir4p complexes, which then deactylate the next nucleosome. This process is repeated many times and propagates the structure of heterochromatin.[3, 4] It stands to reason, then, that there must be some boundary element that serves as a barrier to this propagation of

heterochromatin, and such barrier elements have been identified between heterochromatin and euchromatin.[2]

DNA is known to be arranged in such a manner that there are regions that are condensed and effectively silenced (heterochromatin) interspersed with regions that are transcriptionally active (euchromatin). In order for this to be the case, there must be boundaries between the active and inactive regions. The first fixed-location boundary elements to be studied were discovered in *Drosophila melanogaster*. Subsequently, Kellum and Schedl developed an assay for boundary activity, protecting a gene from position effects.[5] Alternatively, other boundary assays can measure the ability of an element to block activation when flanked by an enhancer and a promoter. This activity can be differentiated from regular gene silencing because the effect is not seen when this boundary element is placed elsewhere in the DNA.[6]

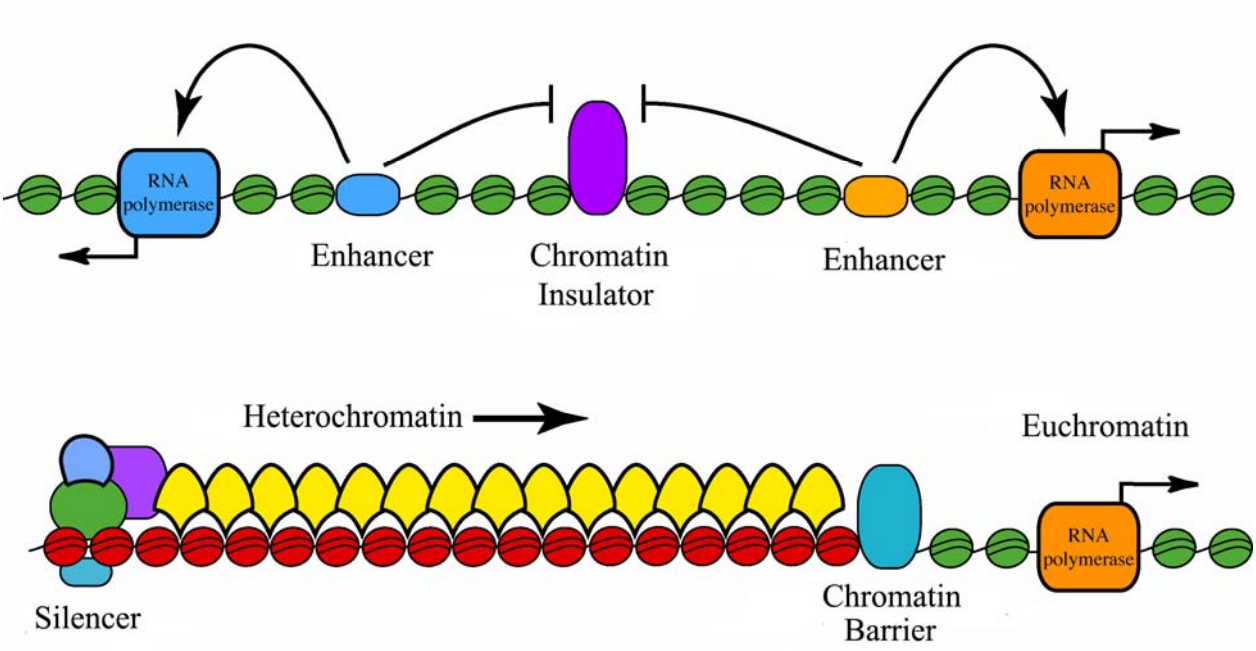

Figure 1.1 - Types of chromatin boundaries in eukaryotes.

Chromatin boundaries in eukaryotes can be classified as either insulators or barriers (Figure 1.1). Insulators are a class of DNA sequence elements that have a common ability to protect genes from inappropriate signals from their surrounding environment. Enhancer blocking prevents enhancer-promoter communication if the insulator is situated between the enhancer and that gene's promoter. This can prevent an enhancer from activating the expression of an inappropriate gene, but leaving it free to affect expression of target genes located on the other side. Boundaries can also act in a second way, which is to prevent the spread of advancing heterochromatin which might silence gene expression. This type of boundary is called a heterochromatin barrier.[7]

Silencing involves the transcriptional inactivation of a large region of a chromosome (usually involving the repression of more than one gene). In the yeast *Saccharomyces cerevisiae* silencing occurs at telomeres and at the silent mating type loci. Yeast exists as two different mating types, mating type '**a**' or mating type '**α**', which are determined by master cell-type specific regulatory genes. The *HML* and *HMR* loci contain cryptic copies of the master **a** and **α** genes which are not expressed. A copy of either one is copied and present at the *MAT* locus. In wildtype yeast, the *MAT* locus contains either one, variably expressed. Most laboratory strains of yeast contain a mutation that will not allow them to switch mating type variably.[2]

At the mating type locus in yeast, the *HMR* locus is silenced by its flanking silencer sequences, which are designated E (essential) and I (important). These silencer elements are composed of autonomously replicating sequences (ARS), that bind the origin recognition complex (ORC), and also of sub-sites that bind the yeast proteins Rap1p and Abf1p (as described previously). These proteins initiate the assembly of a

nucleoprotein complex on the silencer DNA, containing the Sir proteins (Silent Information Regulators, Sir1p, Sir2p, Sir3p, and Sir4p) and other factors responsible for silencing the locus.[2] Downstream of the locus, a discrete boundary demarcating the end of the heterochromatic domain of *HMR* was identified.[8]

Deletion analyses of the right *HMR* boundary indicate that a tRNA gene located downstream of the I silencer can act as a boundary to the propagation of heterochromatin.[9] Deletion of the I element resulted in silencing of an adjacent gene, and ectopic insertion of the I element between the silencer and a reporter gene insulated in the reporter gene from silencing.[6]

There are currently two potential models that work to explain barrier function, passive and active. The passive model suggests that steric hinderance, a physical block to the spread of silencing, would be enough to halt heterochromatin. In this model, any obstruction on the chromosome that would break the deacetylation cycle of the Sir proteins would be enough to inhibit the propagation of heterochromatin. This suggests that any complex that is large enough and bound to the chromosome would then act as a barrier by creating a gap in the nucleosomal array that disrupts the necessary sequential binding of heterochromatin proteins. On the other hand, the active model suggests that where there is a boundary, a stable complex is recruited that either contains within itself or recruits chromatin remodeling enzymes that counter the deacetylation and methylation necessary for the spread of heterochromatin.[2]

The RNA polymerase III complex assembles onto tRNA genes. The complex is comprised of the transcription factor complexes $TF_{III}B$ and $TF_{III}C$, and the 13 subunit RNA polymerase III complex. This complex is large and stable and makes a DNA

footprint of approximately 150 base pairs. Transfer RNA genes contain internal promoters (A box and B box) upon which the $TF_{III}C$ complex assembles.[10-12] When there are mutations in the A box, transcription is decreased because the upstream transcriptional initiation complex cannot be formed correctly. Mutations in the B box (such as mutating an invariant C residue to a G) inhibit binding of $TF_{III}C$ and prevent Pol III complex formation on genes.[13] $TF_{III}C$ binding is required for subsequent binding of $TF_{III}B$, which then recruits RNA polymerase III. The resulting Pol III complex appears to be persistently attached to tRNA genes, as it can initiate multiple rounds of transcription without the need for reassembly. This persistent occupation by the Pol III complex may explain many observed extra-transcriptional roles of tRNA genes.[14]

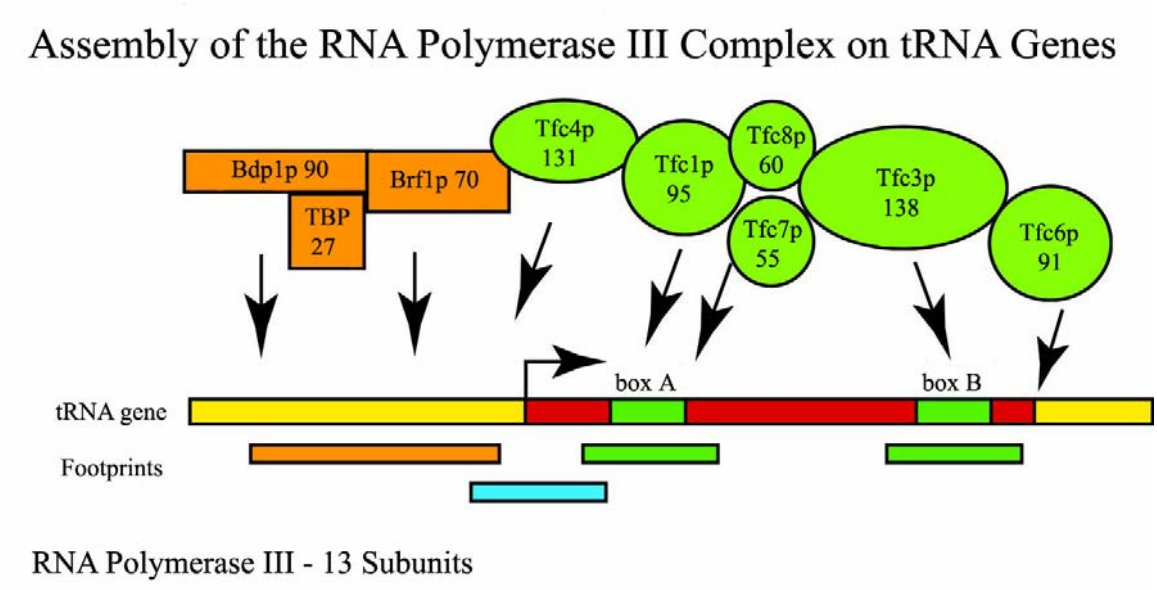

Figure 1.2 - RNA polymerase III transcription factor interactions. Transcription factors depicted in green belong to TFIIIC, which binds to the A box and B box. After $TF_{III}C$ is bound, then $TF_{III}B$ (depicted in orange) can bind to the promoter region and $TF_{III}C$. RNA Polymerase III then bind to $TF_{III}B$ and $TF_{III}C$ and move along the DNA. (Adapted from Geiduscheck and Kassavetis 1992, Huang and Maraia 2001, Paule and White 2000)

Assembled RNA polymerase III (Pol III) complexes are known to exert several extra-transcriptional effects on nearby regions in chromosomes. Ty elements are yeast retrotransposons, and their integration into yeast chromosomes is targeted to regions near actively transcribed RNA polymerase III genes.[15] *S. cerevisiae* contains over 400 tRNA genes (0.1% of the entire genome) which are frequently found near upstream control regions for genes transcribed by RNA pol II. Transfer RNA genes have been found to inhibit transcription from adjacent polymerase II promoters when studied *in vivo*. This effect was shown initially by Sandmeyer by mutating a tRNA gene, leading to increased Pol II transcription of an adjacent Ty element[15], then secondly by Engelke who showed that cloning a tRNA gene adjacent to *HIS3* in yeast resulted in a severe repression of *HIS3* transcription.[13]

There are also several sites, called *ETC* (Extra $TF_{III}C$) loci, conserved within *Saccharomyces* species that bind $TF_{III}C$, but not $TF_{III}B$ or Pol III. This suggests that there may be some function for the bound $TF_{III}C$[16], and we speculate that it may be acting with a boundary function. The $TF_{III}C$ complex is large and could easily block the spread of silencing along a chromosome, thus altering the expression of genes that lie nearby on that same chromosome.

Transfer RNA genes can act as boundaries to the spread of heterochromatic silencing in yeast. Repressed genes are often associated with heterochromatic regions which are characterized by relative hypo-acetylation of histones, and a more condensed chromatin structure. Heterochromatin can propagate along a chromosome and this propagation can be blocked by boundary or barrier elements. At the heterochromatic *HMR* locus in yeast, a specific transfer RNA gene has been shown to act as a boundary to

heterochromatin spreading, providing yet another example of an extra-transcriptional role for RNA polymerase III genes.[14]

Chromosomal experiments show that deleting certain tRNA genes causes expression of an adjacent Pol II transcribed gene to increase[17,18], again demonstrating a negative effect caused by the proximity of the tRNA gene. This is the basis for our systematic analysis of tRNA position effects. To analyze this, we deleted several tRNAs and compared the level of transcription of neighboring genes which are transcribed by Pol II.[17, 19] One possible mechanism to explain this effect is nucleosome positioning. When a tRNA gene is cloned next to a nucleosome positioning sequence, the effect is that the assembled RNA polymerase III complex on the tRNA gene overrides the formation of the nucleosome[20]. Morse *et al.* have shown that replication forks pause at tRNA sites[21]. It has also been proposed that tRNA position effects may be due to nucleolar localization of chromosomal loci containing tRNA genes[22, 23], but we reason that there is an alternative hypothesis that may also explain this phenomenon, insulator-like activity of assembled Pol III complexes.

In the following chapter, we studied the effects of deleting the *TRT2* tRNA gene, whose transcription is not affected by the presence of the α2 operator. This is important because we were looking at the effect on the expression of the neighboring genes *STE6* and *CBT1*, and if the transcription of this tRNA gene was affected by the α2 operator situated between *STE6* and *CBT1*, we would not have been able to get a clear picture of the barrier activity of *TRT2*. The α2 operator is a strong activator, and is mating-type specific. By studying this site, we found that *TRT2* can act as a barrier to repression and exert a position effect on RNA Polymerase II transcription.

We hypothesize that one possible mechanism of the observed tRNA position effects may be due to tRNA genes functioning as insulators, blocking positive signals from the upstream activating sequence of a neighboring gene. Our main goal is to determine how widespread these position effects are on the expression of divergently transcribed genes, and whether or not they are insulator effects from adjacent upstream activating sequences.

The objectives of this thesis include extending the current studies of tRNA position effects, which would involve deleting individual tRNA genes present in the yeast genome and studying each locus individually. As we discovered, some of these genes would need to be expressed through special circumstances. Another objective of this thesis is to study the mechanism of position effects, including insulator effects, boundary effects, as well as other position effects. The third and final major goal for this thesis is to determine how widespread position effects are in the yeast genome. This would involve an extensive study of each tRNA locus in the yeast genome, and eventually testing whether tRNA position effects exist in other types of cells, such as human HeLa cells.

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CHAPTER 2: THE *S. CEREVISIAE TRT2* TRNA^{THR} GENE UPSTREAM OF *STE6* IS A BARRIER TO REPRESSION IN *MAT*α CELLS AND EXERTS A POTENTIAL TRNA POSITION EFFECT IN *MAT*A CELLS*

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INTRODUCTION

RNA polymerase III is predominantly responsible for the transcription of small cellular RNA molecules including tRNAs, 5S RNA, 7SL RNA and in *S. cerevisiae*, the *SNR6* gene encoding the spliceosome U6 RNA. Transcription of tRNA genes is mediated by the stepwise assembly of the $TF_{III}C$ transcription factor complex onto the internal *box* A and *box* B internal control region promoter elements, followed by recruitment of the TBP (TATA binding protein) containing complex $TF_{III}B$. Once all transcription factors are in place, the RNA polymerase III enzymatic complex is recruited to initiate high level transcription of its target genes (1-3). These RNAs are extremely abundant in dividing cells, as tRNAs alone can account for as much as 15% of total RNA in log phase *S. cerevisiae* (4). This number suggests that tRNA genes are transcribed at an amazingly high rate during log phase growth (compared to RNA polymerase II genes), averaging approximately 10^4 transcription cycles/tRNA gene/generation, or roughly twice per second. This high rate of transcription can be explained in part by a facilitated recycling model in which an assembled RNA polymerase III complex is transferred from the termination site to the initiation site, remaining assembled on the tRNA gene through multiple rounds of transcription (5-7).

Such a persistently organized RNA polymerase III complex could also explain several observed "extra-transcriptional" roles of tRNA genes within chromosomes. In *S. cerevisiae*, actively transcribed tRNA genes have been shown to direct Ty element integration (8-10), override nucleosome positioning signals (11), exert repressive position effects on neighboring RNA polymerase II promoters (12-15), act as replication fork pause sites (16), and act as a barrier to the propagation of heterochromatic repression, by blocking the spread of silent chromatin at the *HMR* locus (17). Of particular interest is the dichotomy that in certain cases a

tRNA gene is capable of protecting a neighboring gene from repression (at *HMR*), while in other instances tRNA genes can directly repress or exert a negative influence on transcription of an adjacent RNA polymerase II gene, a process referred to as tRNA-mediated gene silencing (14) or tRNA position effect (15). While these types of effects have been observed in a limited number of cases (both natural and engineered), the genome-wide effects of the location of RNA polymerase III complex formation on neighboring chromosomal loci are largely unstudied.

We have previously described the heterochromatin barrier effect attributed to the *HMR*-tRNA (tRNA^{Thr}[AGU]C) on *S. cerevisiae* chromosome III. This tRNA^{Thr} gene prevents the spread of Sir protein mediated gene silencing from the adjacent *HMR* locus in both reporter constructs and along the native chromosome (17). We asked if tRNAs adjacent to other repressed loci in *S. cerevisiae* could also function as barriers to repression of neighboring genes. *TRT2* (coding for tRNA^{Thr}[CGU]K) is a single copy tRNA^{Thr} gene that lies just upstream of the α2 operator sequence that regulates the *MAT***a** cell specific *STE6* gene on *S. cerevisiae* chromosome XI. We specifically selected this locus for study as another example of a tRNA gene located adjacent to a repressed region of chromatin, and asked whether this tRNA gene might act as a barrier to the spread of repression. The α2 operator binds the Mcm1p/ α2p complex, and initiates *MAT*α cell-specific repression of *MAT***a** specific genes such as *STE6* via multiple mechanisms, including nucleosome positioning (18,19), the recruitment of Ssn6p, Tup1p, and their associated histone deacetylases (20-23). This study asked whether *TRT2* served as a barrier to α2 operator mediated repression in *MAT*α cells, and revealed that the same tRNA gene both protects the adjacent *CBT1* gene from α2 operator repression in *MAT*α cells, and potentially exerts a negative tRNA position effect on *CBT1* in *MAT*a cells. This is the first example of a tRNA gene that displays multiple types of extra-transcriptional functions at the same locus.

MATERIALS AND METHODS

All yeast strains were derived from wild type *S. cerevisiae* W303 (DDY2, DDY3, and DDY4; genotypes of all yeast strains generated in this study are listed in Table 1). Since *TRT2* is an essential single copy tRNA gene, a 0.32 kb fragment of *TRT2* (SGD chromosome XI coordinates 46596-46919) was cloned by PCR into plasmids pRS414 and pRS415 (24) to cover deletions of the gene (plasmids pDD675 and pDD676, respectively). To construct the *trt2-cbt1*Δ::*URA3* reporter strains described in Figure 2.1, a 2.1 kb segment of the *TRT2* locus (coordinates 46162-48248) was amplified by PCR and cloned into pCR2.1-TOPO (Invitrogen) to make plasmid pDD689. The resulting plasmid was cut with *Spe* I and *Xho* I to remove *TRT2* and *CBT1*, and was replaced with the Spe I-Xho I *URA3* fragment from pDD588 (*URA3* cloned into Bluescript SK+) to create plasmid pDD694, *trt2-cbt1*Δ::*URA3*. The modified locus was cut out of pDD694 and transformed into the diploid strain DDY2, and URA+ recombinants were selected and screened by PCR to verify proper integration. This diploid strain was then transformed with *TRT2* plasmids pDD675 or pDD676 to cover the deletion, sporulated, and URA+ haploids were recovered. The *cbt1*Δ::*URA3* control strains were made by direct PCR knockout of *CBT1* with *URA3*, using pRS406 as template. Cells were grown on YMD (yeast minimal medium plus 2% dextrose) lacking uracil to test for repression of the *URA3* marker gene. Yeast Nitrogen Base was purchased from U.S. Biologicals, and YMD plus all mix contained only those nutrients required for growth of W303 strains (adenine, histidine, leucine, lysine, tryptophan, and uracil).

To make the modified chromosomal loci, pDD689 was mutagenized using the Quik-change kit (Stratagene) to delete *TRT2* (oligonucleotides DDO-96/97) from *box* A to the *box* B

(chromosome XI coordinates 46747-46800). The α2 operator from coordinates 46478-46508 was deleted in the same way using oligonucleotides DDO-123/124.

Table 2.1 *S. cerevisiae* W303 strains

		Source
DDY2	*MATα/MAT***a** *ade2-1/ADE2 his3-11/his3-11 leu2-3, 112/leu2-3, 112 LYS2/lys2Δ trp1-1/trp1-1 ura3-1/ura3-1*	J. Rine
DDY3	*MAT***a** *ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*	J. Rine
DDY4	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*	J. Rine
DDY889	*MATα ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2-cbt1Δ::URA3 pTRT2:LEU2*	This Study
DDY890	*MAT***a** *ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2-cbt1Δ::URA3 ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY891	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2-cbt1Δ::URA3 ppr1Δ::HIS3 pTRT2:TRP1*	This Study
DDY902	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2-cbt1Δ::URA3 ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY903	*MATα ade2-1 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2-cbt1Δ::URA3 ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY974	*MATα ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 cbt1Δ::URA3 ppr1Δ::HIS3*	This Study
DDY975	*MAT***a** *ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 cbt1Δ::URA3 ppr1Δ::HIS3*	This Study
DDY1022	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2Δ ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY1024	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2Δ ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY1026	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY1028	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY1261	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ α2 operatorΔ ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY1262	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ α2 operatorΔ ppr1Δ::HIS3 pTRT2:LEU2*	This Study
DDY1737	*MATα ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 α2 operatorΔ*	This Study
DDY1739	*MAT***a** *ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 α2 operatorΔ*	This Study
DDY1740	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 α2 operatorΔ*	This Study
DDY1742	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 α2 operatorΔ*	This Study
DDY1805	*MATα ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2Δ pTRT2:URA3 hos1::HIS3 hos2::TRP1 rpd3::LEU2*	This Study
DDY1825	*MATα ADE21 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ pTRT2:URA3 hos1::HIS3 hos2::TRP1 rpd3::LEU*	This Study
DDY1956	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ ppr1Δ::HIS3 pTRT2:LEU2 hda1Δ::KanMX*	This Study
DDY2021	*MATα ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ ppr1Δ::HIS3 pTRT2:LEU2 hda1Δ::KanMX*	This Study

Plasmids containing deletions of *TRT2* and/or the α2 operator were transformed into DDY889 (*trt2-cbt1*Δ::*URA*), selected on 5-FOA, and proper integration verified by PCR. Resulting strains containing modified *STE6-CBT1* loci were backcrossed to *trt2-cbt1*Δ::*URA3* strains to obtain sibling *MAT***a** and *MAT*α versions.

For Northern blot analysis, RNA was prepared as described in Iyer and Struhl (25). Northern blots contained 10 μg total RNA per lane, and were performed using Northern Max reagents (Ambion). *CBT1* Northerns were run on 1.0% agarose gels, and the *TRT2* blot in Figure 2.4 was run on a 1.2% agarose gel. Northern probes were generated from PCR products of the first 600 bp of each gene (except for *TRT2*, where the entire gene was amplified) that included a T7 RNA polymerase promoter attached to the downstream primer. These PCR products were used as templates to synthesize radiolabeled riboprobes using the Ambion Strip-EZ kit. All oligonucleotide sequences used for knockouts, PCR clonings, probe templates, and mutagenesis reactions are available on request.

HDA1 deletion in the *trt2*Δ strain was made by standard PCR knockout protocols using the plasmid pUG6 as a template (26). The *hos1 hos2 rpd3* strains were made by crossing *trt2*Δ strains with strain DY6445 (*MAT@ ade2 can1 his3 leu2 trp1 ura3 hos1:HIS3 hos2:TRP1 rpd3:LEU2)*, a gift from David Stillman.

Chromatin immunoprecipitation was performed as described in Kuo and Allis (27). Antibodies used were anti-acetyl-histone H3 and anti-acetyl-histone H4 from Upstate (cat. # 06-599 and 06-866). Five μl of a 1:10 dilution of DNA recovered from the immunoprecipitates was used to program PCR reactions (*Taq* polymerase purchased from Promega), and the same volume of a 1:40 dilution was used for the input controls. PCR

conditions were 95C for 2 minutes (initial denaturation), 95C X 30 seconds, 55C X 30 seconds, 72C X 60 seconds (28 cycles).

RESULTS

TRT2 Can Protect an Integrated *URA3* Marker Gene from α2 Operator Repression

STE6 is a *MAT*a-cell specific gene that is repressed in *MAT*α-cells by an upstream α2 operator sequence. Several α2 operator sequences, including this particular one, have been shown to be orientation independent in plasmid based *lacZ* reporter gene assays (28), so we wished to determine if repression was also bi-directional in a chromosomal context. Also, since the *TRT2* $tRNA^{Thr}$ gene lies between this α2 operator and *CBT1*, the next RNA polymerase II transcribed gene upstream of *STE6*, we tested whether *TRT2* acts as a barrier to repression of *CBT1*.

To test the hypothesis that repression spreads bi-directionally from a chromosomal α2 operator, and that the *TRT2* gene acts as a barrier to α2 operator mediated repression, we constructed yeast strains that contained *URA3* integrated in chromosome XI in place of *CBT1*, upstream of the α2 operator site at the *STE6* locus. Two sets of strains were constructed (Figure 2.1A), one that retained *TRT2* between the α2 operator and *URA3*, and a second that replaced both *CBT1* and *TRT2* with *URA3*. Figure 2.1 shows the results when these strains were streaked on minimal media lacking uracil. *MAT*α *trt2-cbt1*Δ*::URA3* strains (Figure 2.1B, DDY 902 and DDY 903, wedges A and B) are considerably compromised for growth on YMD media lacking uracil compared to isogenic *MAT*α (DDY974) or *MAT***a** (DDY975) strains containing *TRT2* between the operator and *URA3* (wedges C and D). *URA3* is not completely repressed in

these strains, as extended incubation eventually leads to formation of colonies. This delay in growth suggests that repression can spread from the α2 operator in both directions along chromosome XI and inhibit *URA3* expression.

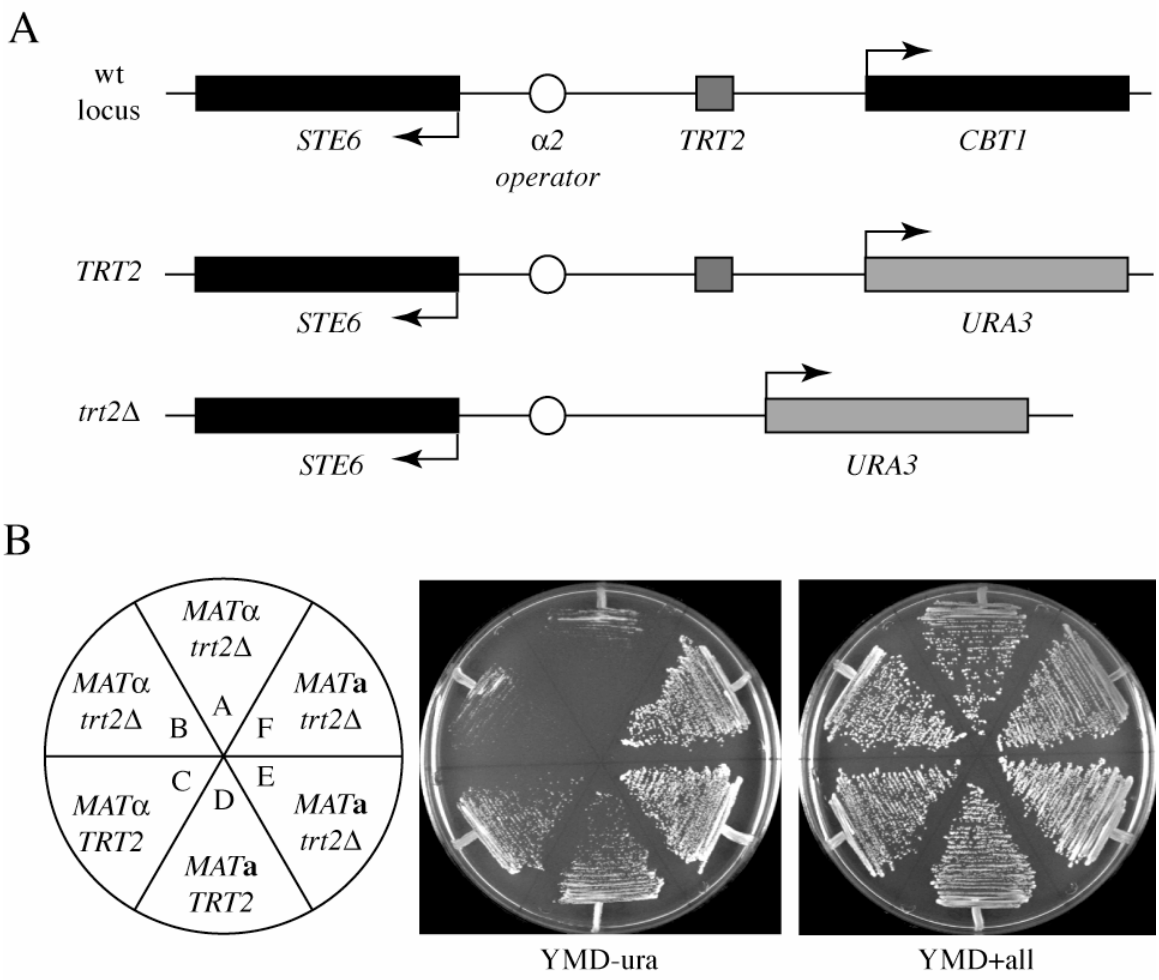

Figure 2.1 - A *URA3* marker gene is repressed when inserted upstream of the *STE6* α2 operator site in *S. cerevisiae* chromosome XI. **(A)** The wild type *STE6-CBT1* region of chromosome XI is depicted on top. *URA3* was inserted by homologous recombination upstream of the *STE6* α2 operator to either delete the *TRT2* $tRNA^{Thr}$ gene (DDY890, DDY891, DDY902, and DDY903), or to retain the intervening *TRT2* gene (DDY974 and DDY 975). **(B)** Each strain was streaked on yeast minimal media (YMD) lacking uracil and incubated for 2 days. *MAT*α strains lacking *TRT2* showed inhibited growth on medium lacking uracil, while all strains grew equally on minimal YMD containing uracil (+all).

Interestingly, *MAT***a** *trt2-cbt1*Δ*::URA3* strains grow slightly better on YMD lacking uracil than *MAT***a** strains containing *TRT2*, suggesting that in the absence of α2 operator mediated repression in *MAT***a** cells, *TRT2* may exert a repressive tRNA position effect on the *URA3* reporter (compare DDY975, wedge D with DDY890 and 891, wedges E and F). These results prompted us to further investigate the effects of deleting *TRT2* on the expression of *CBT1*, the gene naturally upstream of *STE6* on chromosome XI, in both *MAT***a** and *MAT*α cells.

Deletion of *TRT2* from Chromosome XI in *MAT*α Cells Inhibits Induction of *CBT1* When Cells Are Grown on Acetate, and Inhibition Is Dependent on the α2 Operator

CBT1 (Cytochrome B Termination) is a gene required for proper maturation of cytochrome b mRNA in *S. cerevisiae* (29), and is essential for respiratory growth on non-fermentable carbon sources such as acetate and ethanol. *CBT1* is located 862 base pairs upstream of *STE6*, placing it approximately 680 base pairs from the α2 operator. We observed that growth of wild type *MAT*α *S. cerevisiae* in media containing acetate as a sole carbon source (YPAc) resulted in a three-fold induction of *CBT1* mRNA compared to cells grown in dextrose (YPD, Figure 2.2, compare lanes 1 versus 2). We then asked whether *CBT1* expression is affected by deletion of *TRT2*. Since *TRT2* is an essential single copy tRNA gene, it was first deleted in a diploid strain, the deletion was covered with an episomal copy of *TRT2* (pDD676, pRS415:*TRT2:LEU2*), and the resulting diploid strain was sporulated to obtain *MAT*α *trt2*Δ:p*TRT2:LEU2* cells. Deletion of *TRT2* from chromosome XI in *MAT*α cells reduced both the basal and induced levels of *CBT1* expression to approximately 40% of normal levels as analyzed by northern blot analysis (Figure 2.2, lanes 3 and 4, 5 and 6 compared to lanes 1 and 2). This repression was

dependent on the α2 operator, as deletion of both *TRT2* and α2 operator sequences restored the normal levels of *CBT1* mRNA induction (Figure 2.2, lanes 7 through 10). This result demonstrates that repression spreads along chromosome XI upstream of the α2 operator in the absence of *TRT2*, suggesting that *TRT2* functions as a barrier to α2 operator mediated repression of *CBT1* in *MAT*α cells.

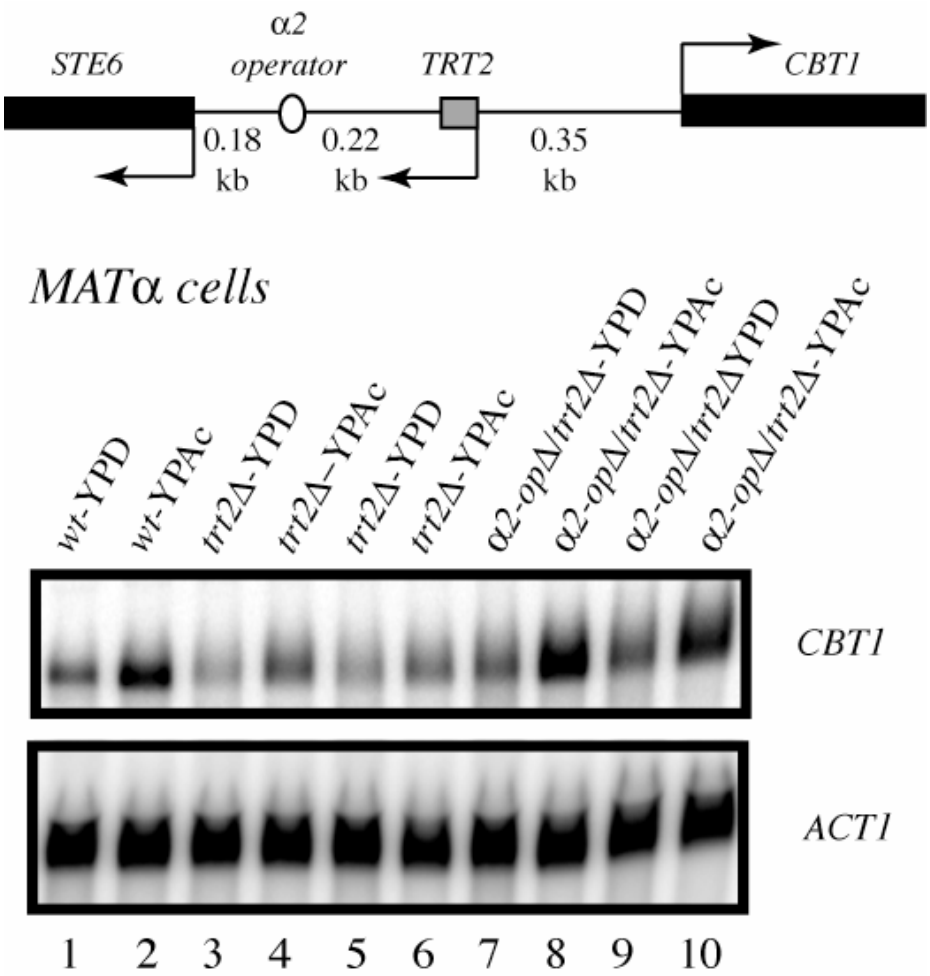

Figure 2.2 - Deletion of *TRT2* results in the repression of *CBT1* transcription in *MAT*α cells. Total RNA was isolated from strains containing a wild type *STE6-CBT1* locus (DDY4, lanes 1 and 2), a mutant locus deleted for *TRT2* (DDY1026, lanes 3 and 4, DDY1028, lanes 5 and 6), and a mutant locus containing deletion of both *TRT2* and the α2 operator (DDY1261, lanes 7 and 8, DDY1262, lanes 9 and 10). Odd numbered lanes contain RNA isolated from cells grown on dextrose as a carbon and energy source (YPD), and even numbered lanes from cells grown on acetate (YPAc), which induces *CBT1* transcription. *CBT1* mRNA levels were reduced approximately three-fold in strains lacking only *TRT2*. Results from two independent isolates of each mutant strain are shown.

Deletion of *TRT2* from Chromosome XI in *MAT*a Cells Results in an Increase in Expression of *CBT1*

When *CBT1* expression from a *trt2*Δ chromosome was analyzed in *MAT***a** cells, the opposite effect was observed. Figure 2.3 shows the results of northern blot analysis of wild type and *MAT***a** *trt2*Δ strains probed for *CBT1* message. Deletion of *TRT2* in *MAT***a** cells leads to increased levels of *CBT1* mRNA in either YPD or YPAc media, suggesting that in its native context in *MAT***a** cells, *CBT1* may be subject to a tRNA position effect (Figure 2.3, compare lane 1 to lanes 2 and 3, lane 4 to lanes 5 and 6). The increased level of transcription of *CBT1* in *trt2*Δ strains is consistent with observation of the strains analyzed in Figure 2.1, as *MAT***a** *trt2-cbt1*Δ::*URA3* strains grew slightly better than *MAT***a** *cbt1*Δ::*URA3* strains on YMD-uracil media.

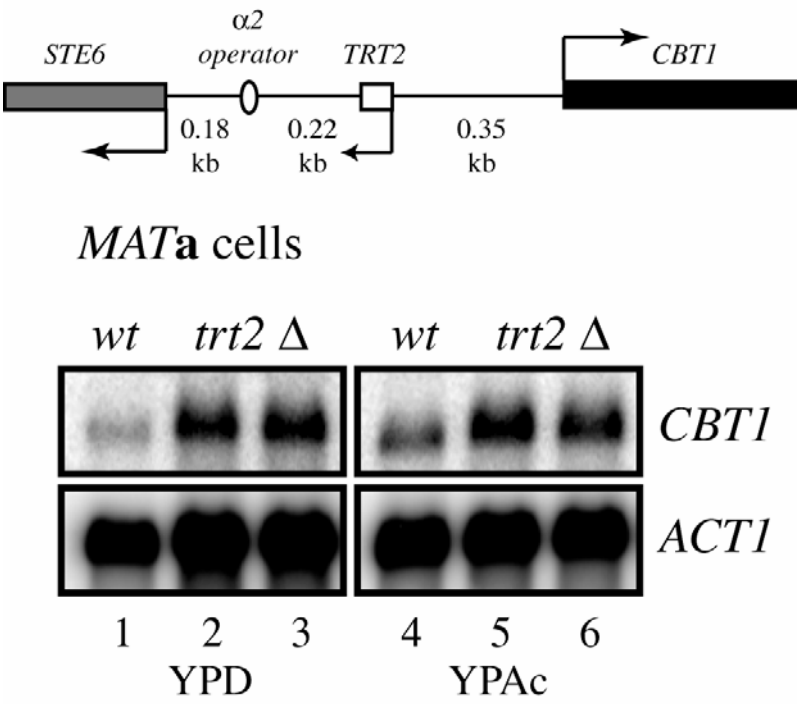

Figure 2.3 - Deletion of *TRT2* results in an increase in expression of *CBT1* in *MAT***a** cells. Wild type *MAT***a** *S. cerevisiae* (DDY3, lanes 1 and 4), and *MAT***a** *trt2*Δ (two independent isolates, DDY1022 lanes 2 and 5, and DDY1024 lanes 3 and 6), were grown on YPD (lanes 1-3) or on YPAc (lanes 4-6) and total RNA isolated. Northern blots were probed for *CBT1* mRNA as in Figure 2.

Transcription of *TRT2* is Unaffected by α2 Operator Mediated Repression

Since *TRT2* is a single copy tRNA gene, its expression level can be assayed directly by Northern blotting. We next asked if the α2 operator affects expression of *TRT2* itself. Figure 2.4 shows *TRT2* expression levels in wild type and α2 operator deleted *MAT***a** and *MAT*α strains. After normalization to the *ACT1* signal, no significant difference in the level of *TRT2* RNA was seen in *MAT*α versus *MAT***a** cells, therefore *TRT2* is apparently unaffected by the presence of an adjacent active α2 operator (Figure 2.4, lanes 1 and 2). To further confirm that the *TRT2* gene is refractory to α2 operator

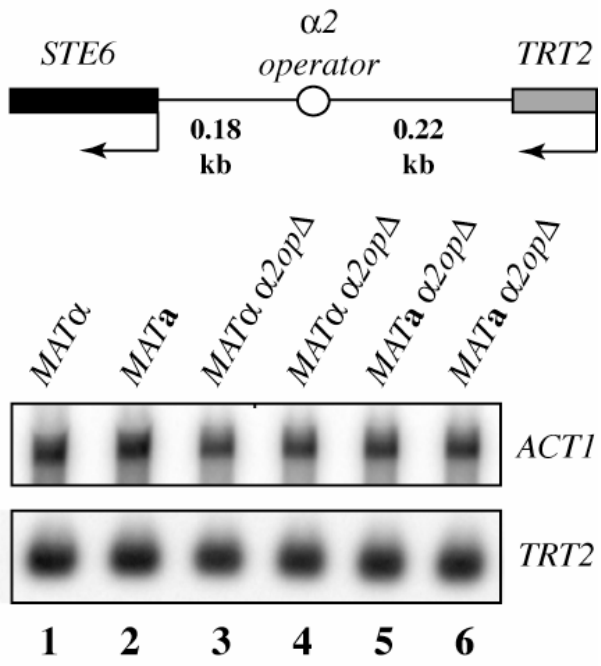

Figure 2.4 - *TRT2* expression is unaffected by the presence of an active α2 operator site. Northern blot analysis of *TRT2* mRNA from wild type *MAT*α and *MAT***a** strains (DDY4 and DDY3, lanes 1 and 2), α2 operator deleted *MAT*α strains (DDY1737 and DDY1742, lanes 3 and 4), and α2 operator deleted *MAT***a** strains (DDY1739 and DDY1740, lanes 5 and 6). After normalization to the *ACT1* signal, *TRT2* mRNA levels were identical in all strains.

repression, the operator site was deleted in both *MAT*α (Figure 2.4, lanes 3 and 4) and *MAT***a** (lanes 5 and 6) strains, and again no difference in *TRT2* levels was seen. These results demonstrate that RNA polymerase III transcription of *TRT2* is completely impervious to α2 operator mediated repression.

Altered Histone Acetylation Does Not Appear to Be Responsible for the Spread of Repression along a *trt2*Δ Chromosome

The recent literature has described multiple yeast histone deacetylases as interacting with the Ssn6p/Tup1p complex to repress transcription. Increased histone H4 acetylation at the *STE6* promoter is observed in class I histone deacetylase (HDAC) *rpd3 hos1 hos2* triple mutant strains (23), however, loss of Rpd3p function affects both repression and activation of *STE6* (30). Derepression of Ssn6-Tup1 regulated genes *SUC2* and *MFA2* is observed in triple *rpd3 hos1 hos2* strains (23). Other Ssn6-Tup1 regulated genes, such as *ENA1* appear to require the class II HDAC *HDA1* for repression, and it has been reported that *STE6* is partially derepressed in either *hda1* or *rpd3* strains (21). The Ssn6-Tup1 protein complex has been shown to physically interact with all of these HDACs *in vitro* (21-23).

To assess whether HDAC recruitment by Ssn6-Tup1 at the α2 operator is responsible for *CBT1* repression in the absence of *TRT2*, we performed Northern blots in *trt2*Δ strains mutated for either *hda1* or *hos1 hos2 rpd3*. Figure 2.5A shows that deletion of *hda1* does not relieve repression of *CBT1* in a *trt2*Δ background. The triple deletion of the class I HDACs results in even lower levels of *CBT1*, suggesting that, as for *STE6* and other genes, *RPD3* function is also required for normal activated expression (30). These results suggest that altered histone acetylation levels are not the major determinant in spreading of repression from the operator in the absence of *TRT2*.

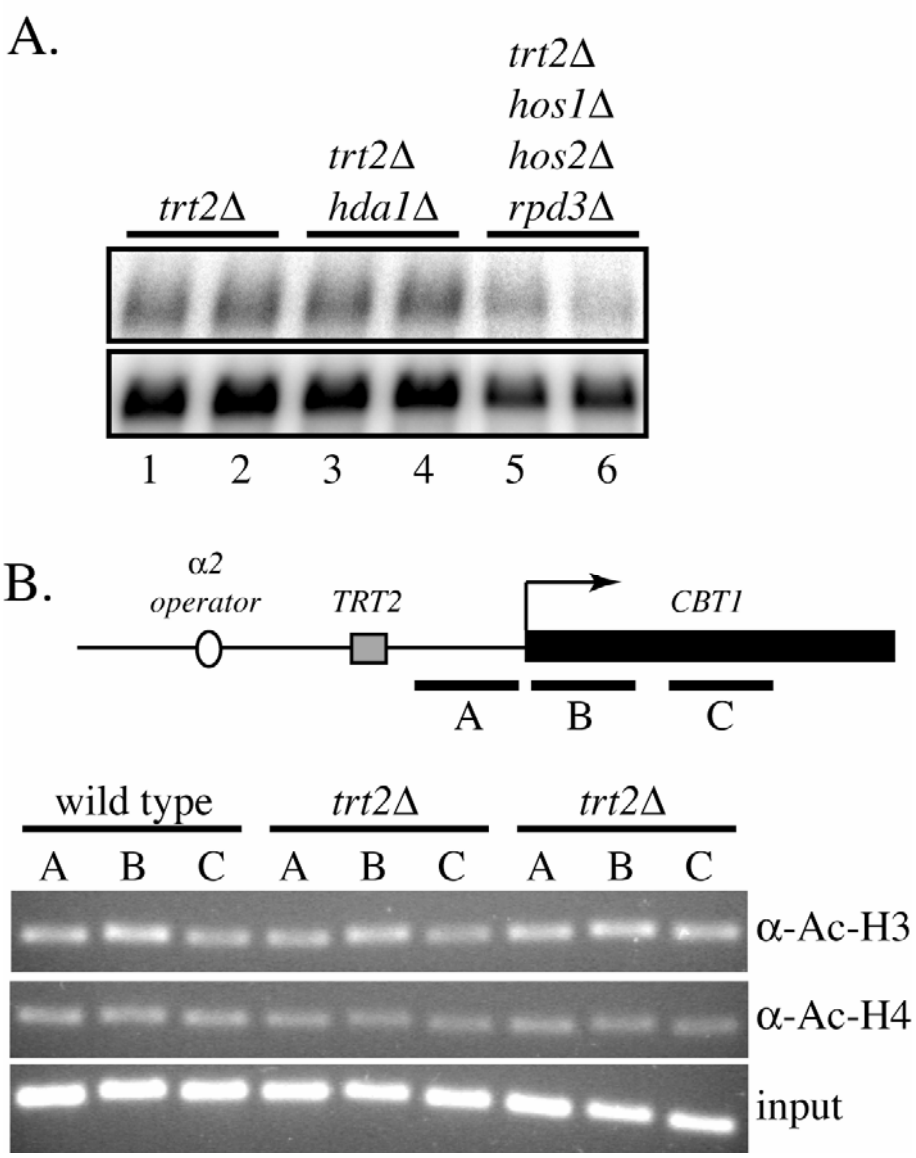

Figure 2.5 - A) Repression of *CBT1* in *trt2*Δ strains is not relieved by mutation of histone deacetylases. Northern blot analysis of *CBT1* mRNA from *MAT*α *trt2*Δ cells containing histone deacetylase mutations. Lanes 1 and 2, *trt2*Δ (DDY1026 and 1028); lanes 3 and 4, *trt2*Δ*hda1*Δ (DDY1956 and DDY2021); lanes 5 and 6, *trt2*Δ *hos1*Δ*hos2*Δ*rpd3*Δ (DDY1805 and DDY1825). B) Chromatin immunoprecipitation of wild type and *trt2*Δ strains using anti-acetylated histone H3 and H4 antibodies. *MAT*α strains DDY4 (wild type) and *trt2*Δ (DDY1026 and DDY1028) were grown and processed for chromatin immunoprecipitation. Primers sets for PCR analysis spanned the indicated regions (approximately 200 bp each PCR producr) of the *CBT1* gene. No significant difference in the level of *CBT1* chromatin was seen in immunoprecipitates from wild type versus *trt2*Δ strains.

In order to directly assess the histone acetylation state at *CBT1* in wild type and *trt2*Δ strains, we performed chromatin immunoprecipitation of the *CBT1* gene using antibodies against acetylated histone H3 or histone H4. Immunoprecipitated DNA was probed by PCR with multiple primer sets spanning from –170 to +500 base pairs from the *CBT1* start codon.

The data in Figure 2.5B showed no significant difference in the amount of immunoprecipitated chromatin between wild type and *trt2*Δ strains. These results also suggest that changes in histone acetylation are not the major determinant in repression of *CBT1* in the *trt2*Δ background, and that other mechanisms of Ssn6-Tup1 repression, either nucleosome positioning or direct interaction with the transcriptional machinery, are responsible (see discussion).

DISCUSSION

α2 Operator Mediated Repression is B*i-Direct*ional at the *STE6* Locus

α2 operator sites mediate repression of transcription of *MAT***a**-cell specific genes in *MAT*α cells (20), and also regulate recombination enhancer activity in mating type switching (31,32). Transcriptional repression is mediated by binding of the α2/Mcm1p complex to the operator sites, which then recruit co-repressors such as the Ssn6p/Tup1p complex. Transcriptional repression by α2 operator sequences is mediated by the further recruitment of various histone deacetylases by Ssn6p/Tup1p (21,23), and by the precise stable positioning of nucleosomes at the promoter region of the regulated gene (18,19,33). Despite a degree of asymmetry of natural α2 operator sites in Mcm1p/α2 regulated genes, cloned α2 operators in either orientation are able to repress transcription of plasmid based reporter genes (28), suggesting that repression can spread bi-

directionally from an α2 operator. This observation led us to analyze whether repression from the α2 operator upstream of the *STE6* gene spreads bi-directionally on the native chromosome, and whether the *TRT2* $tRNA^{Thr}$ gene upstream acts as a barrier to such repression.

The results shown in figures 2.1 and 2.2 show that the *STE6* α2 operator can partially repress upstream genes specifically in *MAT*α cells in a *URA3* modified, or native chromosome XI. The results from the northern blot analysis of *CBT1* mRNA in *trt2*Δ strains shows a 3-fold repression compared to wild type cells. This repression is clearly due to the operator sequence, as its deletion restores the both basal and induced levels of *CBT1* transcription (Figure 2.2). One reason for the relatively mild repression (as compared to the complete repression of *STE6* in *MAT*α cells) could be due to the relative distance between the operator and the gene. The *STE6* gene starts 182 base pairs (bp) from the operator, while the *CBT1* gene is 650 bp away (598 bp in the *trt2*Δ strain). This increased distance may lead to weaker repression compared to that of *STE6*. The range of repression at this locus is limited to the *CBT1* promoter, as deletion of *TRT2* had no effect on expression of *YKL207W*, the next gene centromere proximal to *CBT1* (Donze lab, unpublished). Another possible reason for the relatively mild repression is the asymmetric nature of the *STE6* α2 operator site, which could lead to differences in repression in each direction. A plasmid-based *lacZ* reporter gene was differentially repressed by opposite orientations of this operator, with the native orientation showing 1.5 fold higher repression then the reverse orientation (28). This asymmetry may lie in an asymmetry of direction of Hda1p activity from the operator, which has been proposed for

the *ENA1* promoter (21). Most likely, both distance and orientation are affecting the level of repression of *CBT1* compared to *STE6*.

***TRT2* Acts as a Barrier to Repression**

Since *MAT*α cell specific repression of *CBT1* is observed only when the *TRT2* gene is deleted (or contains only a *box* B point mutation, Donze lab unpublished), *TRT2* is acting as a barrier to the spread of α2 operator mediated repression. We have previously shown that the *HMR*-tRNA ($tRNA^{Thr}$[AGU] CR1) acts as a barrier to the spread of silencing at the *HMR* locus, as it blocks repression of a *MATa1* reporter gene when juxtaposed between the gene and the silencer, and its deletion from the chromosome leads to a 60% reduction of expression of the downstream *GIT1* gene (17). When tested alongside the *HMR*-tRNA in the *MATa1* reporter gene assay, *TRT2* showed a partial barrier activity to Sir protein mediated silencing (17), while it appears to completely prevent the spread of α2 operator repression in this study. Therefore different tRNA genes may vary in their ability to block repression, or may have evolved specificities for different types of repression.

The upstream spread of repression from the α2 operator into *CBT1* does not appear to be mediated by major changes in histone acetylation, as suggested by the data in Figure 2.5. Deletion of HDACs known to be involved in Ssn6p-Tup1p mediated repression do not result in derepression of *CBT1* in *trt2*Δ strains, and chromatin immunoprecipitation with antibodies against acetylated histone H3 or H4 show no difference in the amount of *CBT1* DNA immunoprecipitated in wild type versus *trt2*Δ MATα strains. However, it may be that specific histone deacetylation events may be responsible, which would require a detailed analysis with antibodies specific for

individual acetylated residues. Tup1p has been shown to utilize multiple mechanisms to repress transcription including recruitment of HDACs (21-23), inducing the stable positioning of nucleosomes (18,19,34), and also by direct interaction with the transcriptional machinery (35-37). The results presented here suggest that the latter two mechanisms of Tup1 transcriptional inhibition are most likely at work in the repression of *CBT1* observed in the absence of *TRT2*. Since active tRNA genes have been demonstrated to override nucleosome positioning signals (11), we suggest that the barrier activity of *TRT2* is at least in part due to an ability to block the spread of phased nucleosomes emanating from the α2 operator.

In the Absence of Repression, Deletion of *TRT2* Results in Elevated *CBT1* mRNA Levels

Transfer RNA genes in *S. cerevisiae* have been shown to exert a phenomenon referred to as either tRNA mediated gene silencing or tRNA position effect. In the limited number of cases studied so far, a tRNA gene can exert a repressive effect on transcription from a nearby RNA polymerase II promoter, and this repression requires a transcriptionally active tRNA gene, or at least one competent to bind $TF_{III}C$ (13-15). The genome-wide extent of tRNA position effects is unknown, as it has previously only been observed at a single native chromosomal locus, *PTR3*. However bioinformatic analysis suggests that tRNA position effects may exert a modest but general effect on nearby RNA polymerase II promoters at many loci, and has been suggested that position effects may regulate expression of genes that are derepressed when tRNA expression is downregulated (15). The results shown in Figure 2.3 demonstrate that deletion of *TRT2* increases *CBT1* expression in *MAT***a** cells, where α2 operator mediated repression is

absent. This provides a second potential example of a tRNA position effect on a native gene, supporting the bioinformatic predictions.

It should be noted that the mechanism of tRNA position effects has not been studied in detail on native chromosomal genes. Active tRNA genes have been shown to be localized to nucleoli in *S. cerevisiae* (38,39), and a mutation in the putative psuedouridine synthetase gene *CBF5* disrupts both nucleolar localization of tRNA synthesis and suppresses tRNA mediated gene silencing of a plasmid based reporter gene (14). These studies have suggested that nucleolar localization may be responsible for both tRNA barrier function and tRNA position effects, however, other possibilities exist. One could speculate that inactivation of a tRNA gene could allow upstream activating sequences (UAS) from neighboring genes to inappropriately influence transcription of tRNA proximal genes, suggesting that a tRNA (or an engaged RNA polymerase III complex) might function somewhat as a classic metazoan insulator element, blocking the positive signal from the UAS.

***TRT2* Transcription is Completely Resistant to the Presence of the α2 Operator**

Since the *box* B promoter element of *TRT2* lies only 240 bp from the *STE6* α2 operator, we wanted to ask if transcription of *TRT2* itself was affected by its proximity to the repressive element. The results in figure 2.4 show that *TRT2* is unaffected by the presence of an active (*MAT*α or inactive (*MAT***a**) α2 operator, or by deletion of the operator in *MAT*α cells. Therefore in even in the presence of a nearby active operator site, a fully functional RNA polymerase III complex can form on the *TRT2* gene and carry out normal levels of transcription. This suggests a hierarchy in the assembly of the RNA polymerase III complex onto a chromosome versus the assembly and propagation

of repressive structures, and such a hierarchy may shed some light onto one aspect of the mechanism of the barrier activity of tRNA genes.

Working models of RNA polymerase III transcription depict the stepwise assembly of the $TF_{III}C$ transcription factor complex onto the *box* A and *box* B sites, followed by the recruitment of $TF_{III}B$ proteins Brf1p, Bdp1p, and TBP. Once assembled, this transcription factor platform is able to recruit the RNA polymerase III enzyme complex and initiate transcription (3), in a process that no longer requires $TF_{III}C$. This sequence of events was determined largely from *in vitro* reconstitution experiments, but recent *in vivo* studies suggest a slightly different mechanism.

Chromatin immunoprecipitation studies of human cells progressing through mitosis show that as RNA polymerase III transcription decreases during mitosis, Bdp1 and polymerase subunits are mostly released from chromatin, but Brf1 and TBP remain associated with both tRNA and 5S genes (40). Studies in yeast cells during stationary phase or nutrient limited growth, conditions where RNA polymerase III transcription is markedly reduced, show that polymerase occupancy at a tRNA promoter is severely reduced, while $TF_{III}B$ subunit occupancy is only partially reduced (41,42). Interestingly, these studies show that the association of $TF_{III}C$ appears unchanged or even increased under conditions of reduced tRNA transcription. These results suggest a persistent association of at least part of the RNA polymerase III machinery with its target loci independent the transcriptional state of the gene. This partial association of RNA polymerase III transcription factors is also seen at *ETC* loci (extra $TF_{III}C$), which appear to have $TF_{III}C$ constitutively bound in the absence of $TF_{III}B$ and polymerase (43). The persistent association of RNA polymerase III factors may in one sense serve as an

"epigenetic mark" of these loci for polymerase reassembly when changing conditions require the resumption of RNA polymerase III transcription. Such persistent "marking" of RNA polymerase III promoters may also relate to their barrier function, as it would allow a preferential reassembly of the RNA polymerase III transcription complex after replication, even if the promoter lies adjacent to silencers or other repressive operator elements.

Another feature of RNA polymerase III that may contribute to barrier function is a process called facilitated recycling. Stably bound RNA polymerase III complexes are known to direct multiple rounds of transcription *in vitro* (44,45), and an individual enzyme complex appears to be able to recycle multiple times on an individual template without the need to reform a preinitiation complex (5-7). Although observed *in vitro*, this hyper-processive and persistent occupation of the RNA polymerase III complex is likely to occur *in vivo* to account for the transcription rate required to produce the large number of tRNA molecules per yeast cell. Such a persistent occupation of tRNA genes during all phases of the cell cycle could contribute to the barrier function of tRNA genes by again physically, and perhaps enzymatically (46) preventing the spread of repressive chromatin. With regard to the data in Figure 2.4, the level of *TRT2* transcription from its single locus is identical with or without an active α2 operator, indicating that *TRT2* is transcribed at normal levels by the RNA polymerase III machinery even when adjacent to repressive chromatin. This suggests that RNA polymerase III complex assembly, function and persistence at *TRT2* is dominant over the encroachment of repressive chromatin structures.

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CHAPTER 3: RESULTS

We learned several things as a result of our experiments in Chapter 2. In the absence of *TRT2*, we find that repression mediated by the α2 operator extends in both directions, thus affecting both *STE6* and *CBT1*. This leads us to the conclusion that *TRT2* is acting as a barrier to repression at this locus in *MAT*α cells. In the absence of α2 operator mediated repression in *MAT***a** cells, *CBT1* mRNA levels in *trt2*Δ strains are increased due to tRNA position effects.

tRNA position effects are described as when actively transcribed tRNA genes have a repressive effect on the transcription of adjacent pol II-transcribed genes[18] and is also referred to as tRNA-mediated gene silencing[22]. There are few models that attempt to explain how this position effect occurs. Kendall *et al.* propose that localization of the tRNA genes to the nucleolus may inhibit transcription of nearby genes transcribed by pol II by sequestering the locus to a region of the nucleus that is depleted in Pol II[22]. Another hypothesis for the mechanism of tRNA position effects is the dominant over-riding of nucleosome positioning induced by Pol III complex assembly. While the truth may encompass aspects of each of the models mentioned, we propose another alternative hypothesis to explain the behavior of tRNA genes and chromosomal gene expression, which is that these tRNA genes may be functioning as insulators.

Experiments in Chapter 2 showed us that deletion or mutation of *TRT2* led to an increase in *CBT1* expression in *MAT***a** cells, a result that is repeated in Figure 3.1 below. If the α2 operator is activating *CBT1* in the absence of *TRT2* in *MAT***a** cells, then according to the insulator hypothesis, deletion of the α2 operator should reverse this position effect. In further experiments (Figure 3.1) we deleted the entire region from the α2 operator to the *TRT2* gene and observed a reduced increase in *CBT1* levels, apparently due to activation by the *STE6* regulatory

sequences. When the α2 operator (or more specifically, the Mcm1 binding sites) is deleted in the context of *trt2*Δ, these levels are again reduced, indicating that the rise in *CBT1* expression is partially due to activation by the α2 operator when *TRT2* is inactivated.

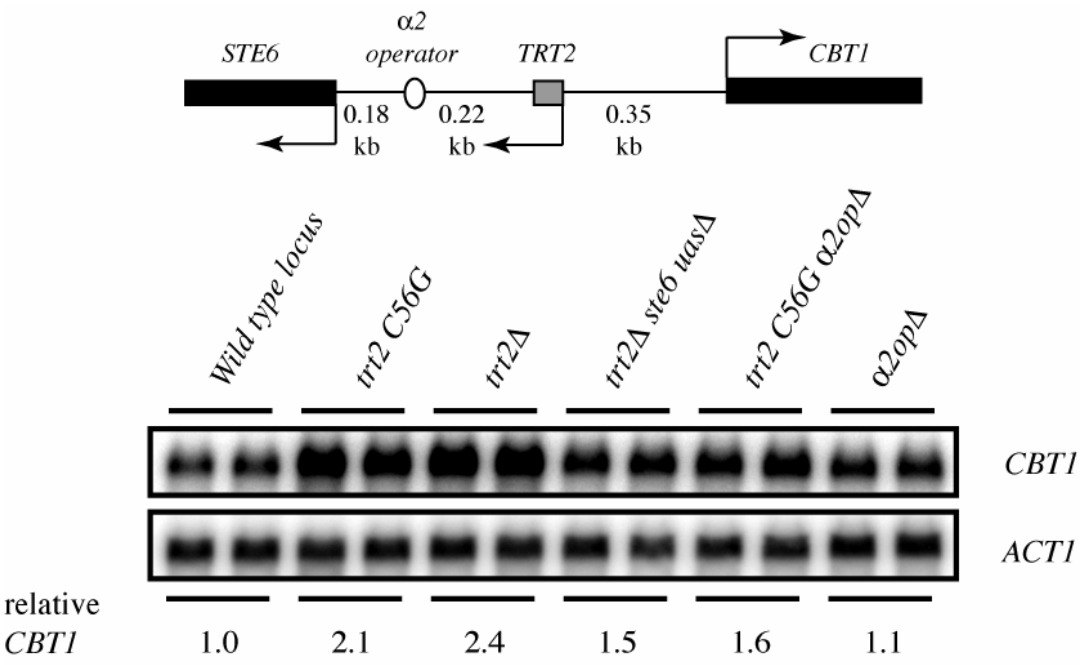

Figure 3.1 – Northern blot analysis of *CBT1* expression in strains containing deletion of the *TRT2* tDNA and *STE6* regulatory elements. Mutation of *TRT2* results in a 2.1-2.4 fold increase in *CBT1* expression, indicative of a tDNA position effect. Further deletion of the *STE6* UAS (from to α2 operator to the *TRT2* gene) or just the α2 operator (only the Mcm1p binding site) reduces this increase by approximately half, suggesting that part of the increase is due to inappropriate activation of *CBT1* by the *STE6* regulatory sequences. Deletion of the α2 operator alone has no effect on *CBT1* transcription. *CBT1* was normalized to *ACT1* levels.

In order to directly test whether or not a tRNA can function as an insulator, we utilized the *GAL1-10* locus. This locus does not normally have a tRNA present, but it is a divergently transcribed pair of genes whose regulation by a common upstream activating sequence (UAS) has been studied extensively. Because *GAL1* and *GAL10* are divergently transcribed, we

inserted a tRNA gene, *TRT2*, between them on either side of the UAS, then mutated the 56th residue from a C to a G which would then make a non-functional tRNA gene without affecting spacing between the UAS and *GAL* genes. We compared the expression of *GAL1* and *GAL10* when they were separated from the UAS by the tRNA gene. In the case of *GAL10*, we see that the functional tRNA completely blocks the activation of *GAL10* when placed between *GAL10* and the UAS. This enhancer blocking activity is abolished when *trt2*C56G was inserted. However, when the tRNA is inserted between *GAL1* and the UAS, *GAL1* expression is lowered, but not to the extent that we see in the case of *GAL10*, and insertion at this location has no effect on *GAL10* expression.

Because tRNA position effects have only been studied at a few select loci, we wanted to determine how widespread tRNA position effects might be. In collaboration with the labs of Giorgio Dieci and André Sentenac we studied tRNA position effects based on microarray analyses. In this analysis, we analyzed Pol II transcription levels in yeast strains containing various temperature sensitive mutations in essential Pol III transcription factors and polymerase subunits, in order to get a genome-wide picture of tRNA position effects. Unfortunately, not all known position effects could be reproduced using this approach, however some Pol II genes did show an effect. The inability of this temperature sensitive mutant approach to provide a comprehensive picture of tRNA position effects was due to global regulatory effects on pol II transcription due to initiation $tRNA^{Met}$ depletion, and due to incomplete inactivation of the temperature sensitive mutations. Based on selected genes whose expression was altered in the microarray analysis, and that were located adjacent to a tRNA gene, we deleted the tRNA gene and directly assayed expression of the neighboring pol II gene (Figure 3.3). This analysis identified position effects at some of the loci studied. *YELO33W* was the only gene that was

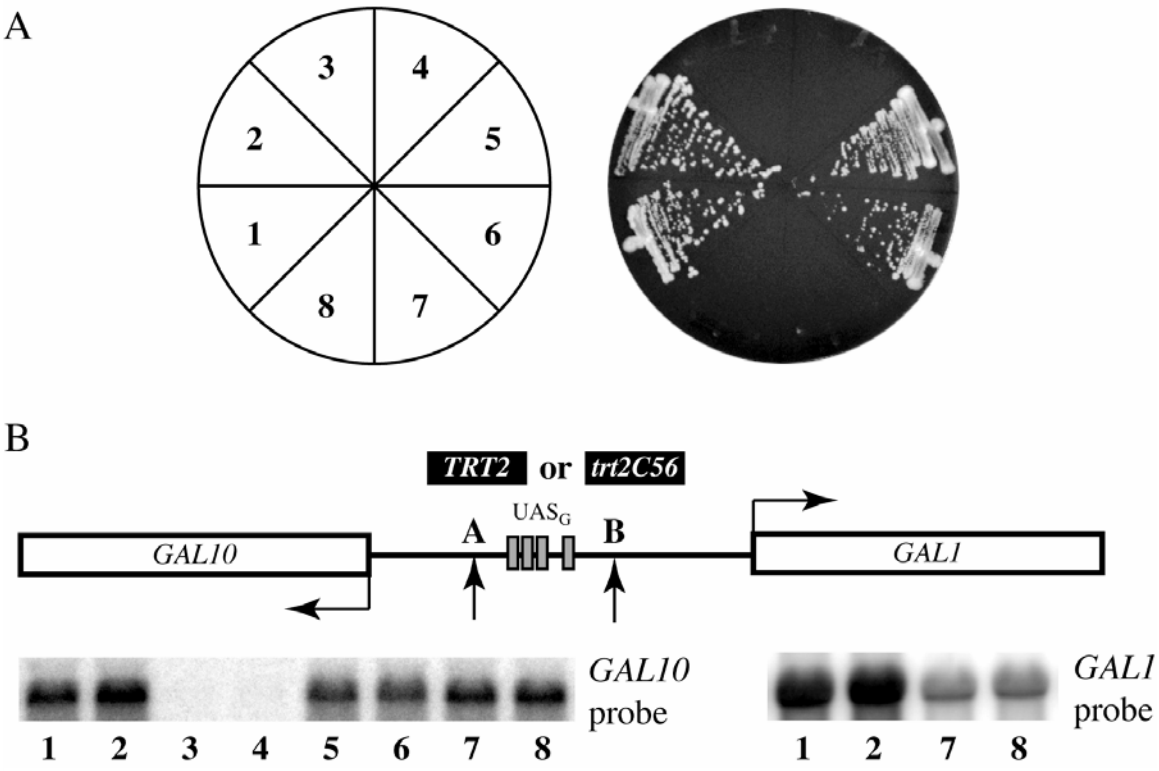

Figure 3.2 - Insulator activity of tDNAs at the *GAL1-10* locus. Functional and mutant tDNA sequences were cloned at various locations within the *GAL1-10* intergenic region and integrated back into the yeast chromosome. A) Resulting strains were streaked onto minimal media with galactose as sole carbon source and grown for 3 days at 30C. B) Cells were grown in raffinose to mid log phase, induced with galactose (2% final concentration) for two hours, then total RNA was isolated and analyzed by northern blotting with *GAL10* or *GAL1* probes. Wedges/lanes 1 and 2, wild type yeast; 3 and 4, *TRT2* inserted between the UAS and the *GAL10* gene (site A); 5 and 6, *box* B mutant *trt2* inserted between the UAS and the *GAL10* gene (site A); 7 and 8, *TRT2* inserted between the UAS and *GAL1* (site B).

consistently shown to be down-regulated, which is consistent with the microarray results. The tRNA gene in this case is downstream of the gene and transcribed in the same direction, whereas for *ACO1*, which has the tRNA gene in the same location but transcribed in the opposite orientation, is up-regulated. *ARO8* is slightly up-regulated upon deletion of the tRNA. *AMD2*, *POR1*, and *YJL200C* showed either inconsistent changes in transcription levels or no changes at all.[19]

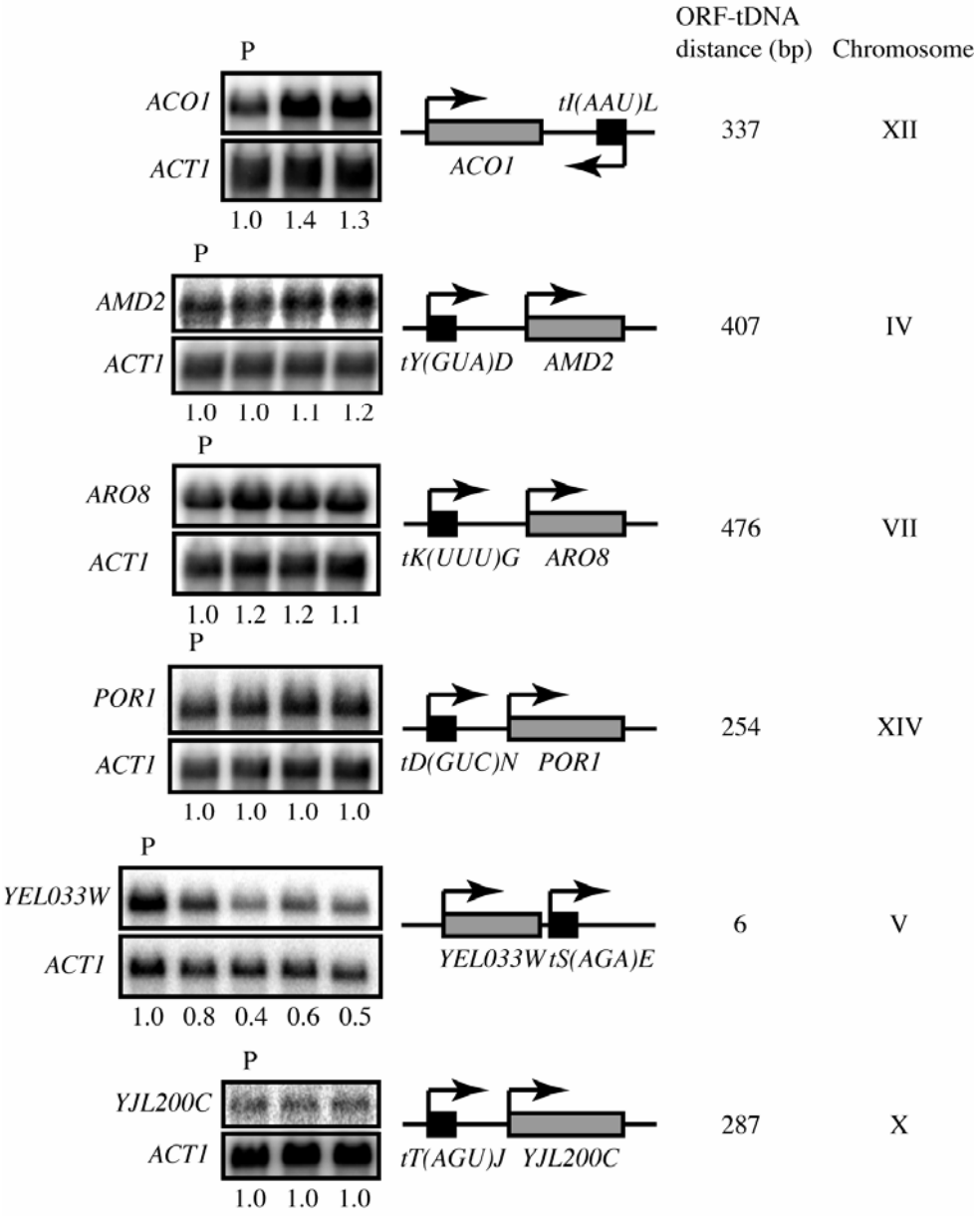

Figure 3.3 - Effects of tDNA deletion on the expression of adjacent Pol II-transcribed genes. The tDNA adjacent to each of the six ORFs (the modified loci are chematically illustrated on the right) was deleted from the chromosome. At least two independent tDNA recombinant strains were isolated in each case. Northern blot analysis of the expression of each Pol II gene is shown compared to the corresponding parent strain (P). Band intensities were determined by phosphorimager analysis, and normalized to the *ACT1* signal for each lane. The values under each lane represent the fold difference of the normalized signals relative to that of the parent strain.
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Since there has been no genome-wide characterization of every tRNA that could potentially be involved in position effects, we systematically searched the yeast genome for divergently transcribed loci which had a tRNA between the genes, as these would be candidate loci where potential position effects might be due to insulator activity. Out of the 69 loci that matched these criteria, we selected five of these divergently transcribed loci to test initially for tRNA position effects. We also selected four out of the seven previously reported *ETC* sites that lie between divergently transcribed loci. For each of these loci we deleted either the tRNA or the *ETC* site and tested for the expression level of the genes present on either side by performing northern blots. When tM(CAU), the tRNA gene that lies between *PEX25* and *CAR1,* is deleted the expression of *PEX25* is decreased whereas the expression of *CAR1* is slightly increased. Deleting tW(CCA) results in a large decrease of *CRH1* and a slight decrease in *HIP1* expression. When tS(GCU) is deleted *TMA10* expression increased in two of the three isolates, while the expression of *NMA1* is decreased in two of the three isolates. These results show that deleting a tRNA can have either positive or negative effects, and that tRNA position effects may be more widespread than previously thought. We deleted *ETC6* and saw a decrease in the expression of *TFC6*, which codes for a subunit of $TF_{III}C$.

Some of these loci did not exhibit a change in expression when comparing the strain with the deleted tRNA or *ETC* site to the parent strain in which the locus was intact. We postulate that either no position effects exist at these loci, or this lack of effects is due to the fact that some of these genes are conditionally expressed. For example, genes involved in the adenine synthesis pathway and are only turned on under conditions of limiting adenine or genes involved in DNA repair are induced under conditions of DNA damage. We are now testing these strains grown under various conditions to look for potential conditional position effects.

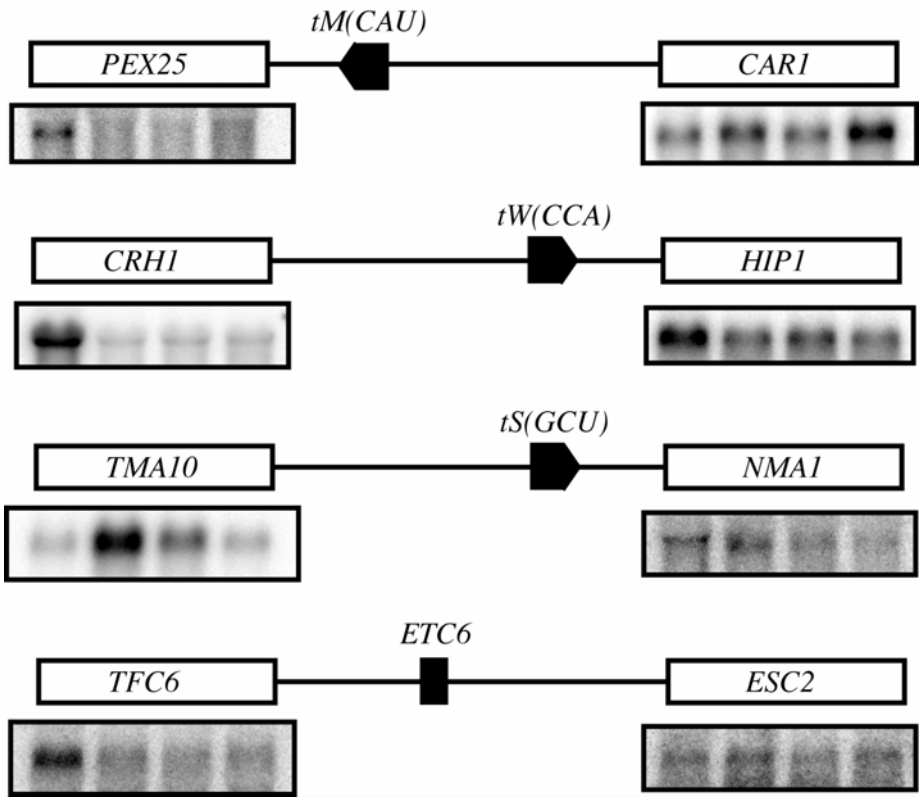

Figure 3.4 – Effect of deletion of tDNA and ETC loci on expression of neighboring genes. For each panel, the first lane contains RNA from the parent strain, and the remaining lanes represent three independent tDNAΔ or ETCΔ isolates. Deletions were made by integrating a loxP-KanMX-loxP cassette to replace each tDNA or ETC6, then excising the cassette by transient expression of Cre recombinase. Northern blots were probed with antisense RNA corresponding to the first 600 nucleotides of the coding sequence of each gene.

DISCUSSION

As was previously discussed, tRNA genes can function as barriers to the propagation of heterochromatin at the *S. cerevisiae HMR* locus. The studies described in this thesis further this idea of bound Pol III factors acting as boundary elements by demonstrating that a tRNA gene can block a different form of repression, the Tup1p mediated transcriptional repression propagating from the α2 operator sequence adjacent to the *STE6* gene. We have been able to show that

certain tRNA genes when located between divergently transcribed genes can also function as enhancer-blocking insulators.

These additional roles of tRNA genes add to a growing list of extra-transcriptional functions of Pol III transcribed genes. Several models have been proposed to describe the mechanism of tRNA position effects. The Engelke lab has shown that tRNA genes can localize to the nucleolus, and hypothesize that this localization can drag adjacent Pol II genes into an environment (the nucleolus) which is unfavorable for Pol II transcription[13]. However, this model may not be universal as it has only been demonstrated using an episomal reporter gene system, and chromosomal position effects cannot entirely be explained by this model. A recent study in *Schizosaccharomyces pombe* has shown similar localization of $TF_{III}C$ to the nuclear periphery[24], another location thought to be associated with pol II gene repression. Our data suggests that there may be an additional mechanism of tRNA position effects, which is mediated by upstream activating sequences of nearby genes. This model postulates that in the absence of a tRNA that lies between divergently transcribed genes, the UAS of one gene inappropriately activates transcription of the other. This activity is extremely similar to metazoan chromatin insulators, which prevent communication between enhancers and promoters. This suggests that tRNA genes have yet another extra-transcriptional role, which is that of a true insulator.

Recent data supports our argument for tRNA genes as boundaries to the spread of heterochromatin. In *S. pombe*, *COC* (chromosome organizing clamp) sites have been identified that appear to be similar to *S. cerevisiae ETC* sites, in that they appear to bind only $TF_{III}C$. These sites generally lie between divergently transcribed genes and have been directly shown to exhibit heterochromatin barrier activity. Their results suggest that *ETC* and *COC* sites act as

chromosomal landmarks, further implicating the possible widespread general boundary effect of $TF_{III}C$ binding sites.[24]

In order to determine how global tRNA position effects may be, a long term study would have to be undertaken in which each tRNA locus in the yeast genome would systematically be studied, starting with those that are divergently transcribed. This would involve creating a collection of yeast strains each deleted for a different tRNA or *ETC* site, and subsequent analysis of transcription of neighboring pol II genes. This would provide a comprehensive picture of the extent and magnitude of tRNA position effects in *S. cerevisiae*.

Given that binding sites for the Pol III complex can have multiple conserved functions in both *S. cerevisiae* and *S. pombe*, analysis of such effects in human cells might provide a ripe field for future studies. This is particularly intriguing when one considers the large number of Pol III transcribed SINEs (short interspersed elements, including the high-copy *Alu* elements) in the human genome. A barrier-like effect has been demonstrated for human *Alu* elements flanking the Keratin 18 gene, as transgenic expression of this gene is reduced when the *Alu* sequences are deleted or mutated to inhibit $TF_{III}C$ binding[25]. If $TF_{III}C$ binding sites are shown to have both barrier and insulator functions in metazoans, their genomic impact may be substantial.

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APPENDIX A: STRAINS

All of the strains used for our initial tRNA study were constructed using a method as described in Cheng/Gartenberg[26]. Oligos were constructed for each tRNA that we wished to delete with homology to the kanamycin gene. The strains constructed then contained the *KAN* gene with loxP sites on either side. Cre recombinase was then used to cut at the loxP sites and excise the *KAN* gene, leaving a single loxP site and completely deleting the tRNA.

tRNA & ETC delete strains

DDY 3067-3069	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	tM(CAU)PΔ	(PEX25-CAR1)
DDY 3070-3072	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	tS(GCU)LΔ	(RBF9-NMA1)
DDY 3073-3075	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	tF(GAA)MΔ	(YMR041C-ARG80)
DDY 3076-3078	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	tN(GUU)O2Δ	(TCB1-YVC1)
DDY 3079-3081	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	tW(CCA)G2Δ	(CRH1-HIP1)
DDY 3082-3084	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	ETC1Δ	(ADE8-SIZ1)
DDY 3085-3087	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	ETC4Δ	(RAD2-TNA1)
DDY 3088-3090	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	ETC6Δ	(TFC6-ESC2)
DDY 3091-3093	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL	can1-100	ETC8Δ	(RPB5-CNS1)

GAL strains

DDY 3	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL can1-100
DDY 2861-2862	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL can1-100 GAL1-10 intergenic wild type replacement
DDY 3256-3258	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL can1-100 gal1-10 intergenic-TRT2 short <--
DDY 3265-3267	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL can1-100 gal1-10 intergeniçtrt2 C56G <--
DDY 3268-3270	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL can1-100 gal1-10 intergenic-TRT2 short -->
DDY 3274-3276	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1	GAL can1-100 gal1-10 intergeniçtrt2 C56G -->

CBT1 strains

DDY 2317	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 pRS415(LEU2)-tRNA KL
DDY 2318	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 pRS415(LEU2)-tRNA KL
DDY 2322	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 trna kl C56G pRS415(LEU2)-tRNA KL
DDY 2323	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 trna kl C56G pRS415(LEU2)-tRNA KL
DDY 2325	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 trna kl Δ pRS415(LEU2)-tRNA KL
DDY 2326	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 trna kl Δ pRS415(LEU2)-tRNA KL
DDY 2329	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 ste6 uas Δ trna kl Δ pRS415(LEU2)-tRNA KL
DDY 2330	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 ste6 uas Δ trna kl Δ pRS415(LEU2)-tRNA KL
DDY 2333	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 α2 operator Δ trna kl C56G pRS415(LEU2)-tRNA KL
DDY 2335	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 α2 operator Δ trna kl C56G pRS415(LEU2)-tRNA KL
DDY 2341	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 α2 operator Δ pRS415(LEU2)-tRNA KL
DDY 2342	MATa	ADE2	his3-11	leu2-3,112	lys2-	trp1-1	ura3-1 can1-100 α2 operator Δ pRS415(LEU2)-tRNA KL

Giorgio Strains

DDY 1821-1823	MATa ADE2 his3 leu2 lys2Δ trp1ura3 ti(uag)l2Δ (ACO1)
DDY 1885-1886, 1896-1897	MATα ADE2 his3 leu2 LYS2 trp1ura3 ty(gua)dΔ (AMD2)
DDY 1903-1906	MATα ADE2 his3Δ1 leuΔD0 lys2Δ0 TRP1 ura3Δ0 tk(uuu)g1Δ (ARO8)
DDY 1899-1902	MATα ADE2 his3Δ1 leuΔD0 lys2Δ0 TRP1 ura3Δ0 trnad(guc)nΔ (POR1)
DDY 1833, 1840-1842	MATa ADE2 his3 leu2 lys2Δ trp1 ura3 ts(aga)eΔ (YEL033W)
DDY 1818-1820	MATa ADE2 his3 leu2 lys2Δ trp1 ura3 tt(agu)jΔ (YJL200C)

APPENDIX B: OLIGONUCLEOTIDES

632-IMT2 5′ KO-ATATAACCTGAAATAATAAGCCCAATCCCACCGGAATTTCATATACTATCACCGACCTGGGCCAGCTGAAGCTTCGTACGC
633-IMT2 3′ KO-AAATATTTTGGTGAATTTGCTCGGTACGCGCTAAACTCACATGATTTAGCGCATAGGCCACTAGTGGATCTG
634-IMT2 5′ check-GTTTCTATAGGTGAAGACTTAC
635-IMT2 3′ check-TAACTACATACAGTGTCGAGG
636-PEX25 T7 probe-TAATACGACTCACTATAGATTTGAAAGTTGCACTTTCTGG
637-PEX25 5′ probe-CAGTTTGGCACGACAGATATC
638-CAR1 5′ probe-GAAACAGGACCTCATTACAAC
639-CAR1 T7 probe-TAATACGACTCACTATAGAACATCTCTCAACCCAATATAC
640-tS(GCU)L 5′ KO-GTGACACAAAATTTGGACAATATAACGATTCATTTTTAGATCGTTGTTCAACGCCAGCTGAAGCTTCGTACGC
641-tS(GCU)L 3′ KO-GTGTTCTATCCTACGTAAGCGGATGCAGCGCAATTCCAGCCGTCTTCATCGTCGCATAGGCCACTAGTGGATCTG
642-tS(GCU)L 5′ check-GCAGAAATTGCGCTATTCCG
643-tS(GCU)L 3′ check-CTGACTCTATTAACTATGAGAAA
644-RBF9 T7 probe-TAATACGACTCACTATAGGATGTGGTATTGTTGCAAATCA
645-RBF9 5′ probe-ACCAGAACTAGCAAATGGACA
646-NMA1 5′ probe-CCCACAAGAGCTCCGGATT
647-NMA1 3′ probe-TAATACGACTCACTATAGTGCTTTTGATAGTTATCACTAAC
648-tF(GAA)M 5′ KO-CATGTATTTCCATGAGAATGGGCTCGCATCCCAGAGCGCAACTAATATATGGCCAGCTGAAGCTTCGTACGC
649-tF(GAA)M 3′ KO-TTACCGTCTAATAATGGATGACCCACCCGCTTCTGTGATGCTGCTCATCACCGCATAGGCCACTAGTGGATCTG
650-tF(GAA)M 5′ check-TCGACTACATATGCACAATGC
651-tF(GAA)M 3′ check-CTTTAGAGAGTGTATGTAACTAA
652-YMR041C T7 probe-TAATACGACTCACTATAGTGCGTTGCAAACCATTTTCAG
653-YMR041C 5′ probe-TAATGAAAAAGTGAATCCATTCG
654-ARG80 5′ probe-CGTCGAATAGCGACGGTTC
655-ARG80 T7 probe-TAATACGACTCACTATAGGTTTGCCGGAGATTGTTCCT
656-tN(GUU)O2 5′ KO-TATCGTCATAATAAGTTCTTCATTCGTTCTCTAAAACAACAAAGTTGCCAGCTGAAGCTTCGTACGC
657-tN(GUU)O2 3′ KO-CCACATATTTTCAGCTCTTATGAGATAACTCCGACATAGCAACAGTGTAGGGCATAGGCCACTAGTGGATCTG
658-tN(GUU)O2 5′ check-GTCCTTTGGTTATTTTTATGTCA
659-tN(GUU)O2 3′ check-AACTGAATTCTATTGCCTTACC
660-TCB1 T7 probe-TAATACGACTCACTATAGTAGTAGCCATTTGTTCATTAGC
661-TCB1 5′ probe-CCAAAGAAGATACTGGGGTAA
662-YVC1 5′ probe-TATCAGCCAACGGCGACTTG
663-YVC1 T7 probe-TAATACGACTCACTATAGCGCCAGTGAATCGTCCTTG
664-tW(CCA)G2 5′ KO-CCATACCACGAAAAGCAAGCCCTCAGAGGTTCTAATGCATTATAGCTCAGATCGCCAGCTGAAGCTTCGTACGC
665-tW(CCA)G2 3′ KO-TCCACGGAATAAGATTGCAATCGAAGGGTTGCAGGTTCAATTCCTGTCCGGCATAGGCCACTAGTGGATCTG
666-tW(CCA)G2 5′ check-CATCCATTGTTGCATGAGGG
667-tW(CCA)G2 3′ check-CAAACACAGAATTTCGGCAGA
668-CRH1 T7 probe-TAATACGACTCACTATAGGGTACATAGGAGATTGTGGG
669-CRH1 5′ probe-GTGCTTGACCTACTAACGGT
670-HIP1 5′ probe-CTAGAAACCCATTGAAAAAGGA
671-HIP1 T7 probe-TAATACGACTCACTATAGGGCATAAAAGATAGCAACCCA
687-ETC1 5′ KO-CTTTCCTCTTGCTGGAATAAAAAACAGGTCAGAGTCACTAGCAACGAGTCAGCCAGCTGAAGCTTCGTACGC
688-ETC1 3′ KO-AATGCATTTTCCTTCCGACTTCGAAGATAGATTTCAGCATAAAGACTAAAAGCATAGGCCACTAGTGGATCTG
729-ETC1 5′ check-CAACCTTGAGGCTAGGAGC
690-ETC1 3′ check-ACATCTAGGGGCCGTTTAGT
691-ADE8 T7 probe-TAATACGACTCACTATAGGCTGTAAAACCTTATATGTAGC
692-ADE8 5′ probe-GAATTGTCGTATTAATTTCGGG
693-SIZ1 5′ probe-TAAATTTAGAGGATTACTGGGAA
694-SIZ1 T7 probe-TAATACGACTCACTATAGTCCCAGAAAATAAGTATAGCCT
695-ETC4 5′ KO-TATATGTGGCGGCATCTTTTCTTTTTCTTTTCTTTTTTTTTTTTTTCACTCTCCCTTTTAATGCCAGCTGAAGCTTCGTACGC
696-ETC4 3′ KO-TTGCTGTTTTTTTTTTTTCATTGTTAATTATCACTATTTTTTTTCTTTCTAGTATAAGCATAGGCCACTAGTGGATCTG
697-ETC4 5′ check-GAAGTTTGGCGTTCTCCCG
698-ETC4 3′ check-AAGTAAGGTTTGCATATGCGG
699-RAD2 T7 probe-TAATACGACTCACTATAGCTTGATTTCAACCTCAACGAC
700-RAD2 5′ probe-GGTGTGCATTCATTTTGGGAT
701-TNA1 5′ probe-GCAACAAATTTACAATGGAGTC
702-TNA1 T7 probe-TAATACGACTCACTATAGGCATATTGTTCTCTTCTATAGC
703-ETC6 5′ KO-CAACTCATCCAGGCTTTCTCGAACAAAAAATGGAATGTTGTTTATCTTCTTTTGCCAGCTGAAGCTTCGTACGC
704-ETC6 3′ KO-ATTTGCTGTCTTCTGTAAGGAAATAGAAGGGATTCAGTATCACCCGGAAAGCTGCATAGGCCACTAGTGGATCTG
705-ETC6 5′ check-ATTATTACACGTATCGCAATGG
706-ETC6 3′ check-CTATTTCAATTGCGATATACGC
707-TFC6 T7 probe-TAATACGACTCACTATAGGGAGAATCAGGCTGAATATTC
708-TFC6 5′ probe-AGTAATACCGGCAAAGAAAAGA
709-ESC2 5′ probe-CGGTGATTCCAGAAGCATCA
710-ESC2 T7 probe-TAATACGACTCACTATAGCGAGGACCTTCACTTGGAC
711-ETC8 5′ KO-ATGATTGAAAATTTTTCAAGAGAAAAGAAAAATTTTTCCTTCCGCGGTCAGCCATTGCTGCCAGCTGAAGCTTCGTACGC
712-ETC8 3′ KO-AGTTAATATGCAAGCATTTAAAATTAAAGTGGCAATGTATTGTTGTTGTCCGCATAGGCCACTAGTGGATCTG
713-ETC8 5′ check-GGCTCGTGATCTGTTGACC
714-ETC8 3′ check-AGACTCTCTACATATAAAGTGC
715-RPB5 T7 probe-TAATACGACTCACTATAGCAATCCCAAGTATAAGGCTAC
716-RPB5 5′ probe-CCAAGAAAATGAAAGAAACATCT
717-CNS1 5′ probe-GCTCCGTTAACGCAAATGGA
718-CNS1 T7 probe-TAATACGACTCACTATAGTCTTTTGCTTTCAATTCTTGTTC

VITA

Tiffany Anne Simms was born on June 29, 1981, to James Simms and Carol Simms, who divorced in 1987. James Simms acquired full custody and raised Tiffany as a single parent while returning to college for a degree in electrical engineering. From an early age, Tiffany was interested in music and wildlife. She started playing the violin at the age of 8, and quickly taught herself any other instruments she could get her hands on. She would also take the time to appreciate nature, often capturing and studying many different creatures. This led her to a love of the sciences. In school, Tiffany often excelled in many subjects. Graduating from Holy Savior Menard Central High School in 1999, she was a part of the first graduating class to have the opportunity to receive the Louisiana TOPS scholarship. This scholarship funded the first four years of her undergraduate degree. After receiving her Bachelor of Science degree in December of 2003 from Louisiana State University, Tiffany worked as a laboratory technician for 7 months before returning for her Master of Science degree, which will be awarded in August of 2006.